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The BRCA1 and BRCA2 genes have been implicated in the DNA repair processes that remediate damage resulting from ionizing radiation, a known risk factor for breast cancer. We have identified and/or acquired 5 established lymphoblastoid cell lines from BRCA1 carriers, 4 from BRCA2 carriers, and 9 control normal lines, all chosen so as to reflect a diversity in age of the individual when the original blood sample was drawn. To date, replicated drug selection clonogenic mutation assays at the X-linked HPRT reporter gene have been completed on 4 BRCA1 cell lines and 5 control cell lines. The average (± standard deviation) mutation frequency of the control cell lines was 11.4 ± 9.0 x 10^{-6}, in good agreement with published studies. The average mutation frequency for the cell lines from BRCA1 carriers was 17.7 ± 3.8 x 10^{-6}, which was not significantly higher than that of the controls (p = 0.23). These data suggest that inheritance of an inactivating mutation in the BRCA1 gene does not inherently lead to an increased frequency of spontaneous mutation, and therefore a lack of DNA repair capacity is not responsible for their predisposition to breast cancer.

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Introduction

About 10% of incident breast cancer can be attributed to hereditary factors, the most important of which are inactivating mutations in the breast cancer predisposing genes BRCA1 and BRCA2. It has been estimated that 75% of human cancer arises due to environmental effects, and this also happens to be the proportion of breast cancer incidence that is not accounted for by hereditary and lifestyle factors associated with endogenous estrogen exposure. Applying the paradigm developed from hereditary non-polyposis colorectal cancer (HNPCC), where loss of function of genes in the base mismatch DNA repair pathway result in mutational susceptibility and subsequent cancer predisposition, we proposed to investigate the possible effects of inherited mutations in the BRCA1 and BRCA2 genes on the spontaneous frequency of mutation in cell lines established from mutation carriers. The BRCA1 and BRCA2 genes have been implicated in the DNA repair processes that remediate damage resulting from ionizing radiation, a known risk factor for breast cancer.

Body

This grant was funded as a concept award, and therefore there was no Statement of Work associated with this grant. We will follow the basic outline of the one-page grant proposal and describe the work that has been accomplished since the grant was funded.

A fundamental characteristic of cancer, including breast cancer, is genetic instability, as evidenced by karyotypic abnormalities and susceptibility to mutagenesis. Genetic instability can be defined and measured as hypermutability; that is genetically unstable cells should either have a high endogenous frequency of gene mutation, or they should be hypersensitive to the actions of known mutagens, or both.

The HPRT mutation assay is a very well established method of measuring gene mutation frequency. It is somewhat labor-intensive because it requires culture and drug selection of viable lymphocytes or cell lines, but it is applicable to all cells and there is a vast literature available for interpretation of the results. Moreover, one class of HPRT mutants have been specifically identified as occurring via illegitimate V(D)J recombination, a mutagenic process that is characteristic of loss of double strand break DNA repair, such as in the cancer-prone syndrome ataxia telangiectasia (AT). BRCA1 and BRCA 2 have also been implicated in this type of repair, so may also have a characteristic increase in these types of mutants.

Hypermutability in the somatic tissue of BRCA1 and BRCA2 carriers would go far to explain the mechanism of their cancer predisposition. If their hypermutability were linked to susceptibility to specific classes of genotoxic agents, for example ionizing radiation and radiomimetic chemicals, efforts could be made to minimize such exposures in known carriers. Alternatively, if loss of heterozygosity at the BRCA1 and BRCA2 genes are necessary for expression of genetic instability, efforts could focus on targeting the preneoplastic cells lacking these genes, and "curing" the disease before it ever becomes manifest.

In the original submission, all cell lines to be used in this study were to be obtained from Dr. Mary-Claire King of the University of Washington. Exempt status for the use of these lines was granted by the University of Pittsburgh Human Subjects Institutional Review Board. Upon funding of this grant through the Department of Defense Breast Cancer Research Program, the DOD IRB required further documentation, such as original consent forms signed by the donors of these cell lines, which Dr. King found excessively burdonsome, and she withdrew from the collaboration. Fortunately, cell lines appropriate for this project have been deposited by various sources in the American Type Culture Collection, and were available for purchase. Although access to these lines allowed for the continuation of this project, it should be stressed that they have a very limited amount of accompanying clinical and demographic data, unlike the cell lines in Dr. King's cell bank, and therefore severely limit our ability to assess any possible associations between mutation frequencies measured in these cell lines and clinical parameters from their donors, such as age at onset of tumor, outcome, etc.

While she was still involved in the project, Dr. King suggested that we investigate whether age matching of patient and control cell lines was necessary by determining whether age at draw affected mutation frequencies in the resulting established cell line. It is well known that in vivo mutation frequencies at both the HPRT gene and others increases significantly with age at draw (Vrieling et al., 1992; King et al., 1994; Akiyama et al., 1995), but it is not clear whether this effect is due to accumulation of mutations over time or whether there is an intrinsic change in the baseline mutation frequency. With this consideration in mind, we have identified and/or acquired 5 established lymphoblastoid cell lines from BRCA1 carriers, 4 from BRCA2 carriers, and 9 control normal lines from the ATCC cell repository, all chosen so as to reflect a diversity in age of the individual when the original blood sample was drawn (**Table 1**).

Controls		BRCA1		BRCA2	
Cell Line	Age	Cell Line	Age	Cell Line	Age
AG09393	4				
GM03797	14				
GM00946	22				
GM05380	32				
AG10111	33	GM13709	33	GM14788	36
GM01814	44	GM13712	43	GM14805	43
GM14820	57	GM13710	57	GM14639	53
GM14448	65	GM13708	68		
		GM13711	75	GM14623	70
AG09980	92				

Table 1. Established lymphoblastoid cell lines available from the ATCC appropriate for analysis in this project.

Due to our extensive experience with the established human lymphoblastoid cell line TK6, we anticipated no problems performing the HPRT assay on a set of such cell lines. We found, however, that culture and drug selection conditions differed somewhat amongst the cell lines, such as in their requirement for a layer of feeder cells. Overall, these cells grew rather poorly in our hands. To date, replicated drug selection clonogenic mutation assays at the X-linked HPRT reporter gene have been completed on 4 BRCA1 cell lines and 5 control cell lines. One control line, AG09980, derived from a 92 year old individual did nor grow well enough in our hands to successfully perform the HPRT assay. This is consistent with the general observation in the field, and reiterated in our own study, that age of donor affects the viability of the resulting cell line ($p = 0.031$ for data from individual experiments). The average (± standard deviation) mutation frequency of the control cell lines completed was $11.4 \pm 9.0 \times 10^{-6}$ (**Table 2**), in good agreement with published studies (for example, Sussman et al., 1999). The mutation frequency in these lines was found to be significantly associated with age of the subject at blood draw ($p = 0.041$ for averaged data, $p < 0.001$ for data from individual experiments), and inversely associated with cloning efficiency, a measure of the viability of cells in the assay ($p = 0.006$ for data from individual experiments) (Cole et al., 1988). Analyzing data from individual experiments (i.e. all six independent results from GM05380, etc.) by multiple analysis of variance revealed that the cell identifier was a significant predictor of mutation frequency ($p = 0.009$), suggesting that each cell line has a uniquely identifiable mutation frequency.

Cell Line	Age	Number of Experiments	Cloning Efficiency	HPRT Mutation Frequency ($X 10^{-6}$) ± SD
Controls				
GM05380	32	6	20.9 %	3.6 ± 1.9
AG10111	33	6	17.4 %	5.8 ± 3.3
GM01814	44	2	8.5%	9.8 ± 5.9
GM14820	57	4	9.7 %	11.0 ± 10.6
GM14448	65	4	10.9 %	26.6 ± 14.8
Average	46.2		13.4 %	11.4 ± 9.0

Table 2. HPRT mutation frequencies of control lymphoblastoid cell lines.

The average mutation frequency for the cell lines from BRCA1 mutant gene carriers was $17.7 \pm 3.8 \times 10^{-6}$ (**Table 3**), which was not significantly different than that of the controls ($p = 0.72$). However, there was no association of mutation frequency with age in these samples ($p = 0.14$ for averaged data, $p = 0.50$ for data from individual experiments), and the trend is towards decreasing mutation frequency with age rather than increasing mutation frequency. Once again, an inverse correlation of mutation frequency with cloning efficiency was observed ($p = 0.052$ for averaged data, $p = 0.002$ for data from indivdual experiments), although the cloning efficiencies of these BRCA1 carrier-derived lines was significantly lower than those of controls ($p = 0.008$).

Cell Line	Age	Number of Experiments	Cloning Efficiency	HPRT Mutation Frequency ($X 10^{-6}$) ± SD
BRCA1				
GM13709	33	2	1.7 %	23.3 ± 16.2
GM13712	43	3	3.9 %	16.6 ± 13.4
GM13710	57	3	4.2 %	16.0 ± 9.5
GM13708	68	3	3.6 %	15.1 ± 12.2
Average	50.0		3.3 %	17.7 ± 3.8

Table 3. HPRT mutation frequencies of BRCA1 carrier-derived lymphoblastoid cell lines.

By dropping control cell line GM05380 from the analysis we can compare the control and BRCA1 carrier-derived lines using a paired t-test for age-matched pairs (a more powerful test of correlation). The ages ($p = 0.81$) and mutation frequencies ($p = 0.51$) of the two populations remain statistically indistinguishable, while the cloning efficiencies remain significantly different ($p = 0.047$). The mutation frequencies of the BRCA1-derived lines are more consistent than those of the controls despite the range in ages at draw; multiple analysis of variance fails to identify the cell identifer as a significant predictor of mutation frequency in this data set. Indeed, only the inclusion of the unusually high mutation frequency from control cell line GM14448 precludes the difference in mutation frequcny in these two sets of cells from being significant ($p = 0.007$). One interpretation might be that the BRCA1 lines express a mutation frequency appropriate for an older individual at a young age which is then not affected by time. Analysis of more cell lines of both types would be necessary to demonstrate such a subtle manifestation of the heterozygous $BRCA1^{+/-}$ genotype.

Key Research Accomplishments

- We have identified and obtained three sets of established lymphobalstoid cell lines from controls, mutant BRCA1 gene carriers and mutant BRCA2 gene carriers from individuals sampled at different ages.

- We have established flexible methodologies allowing for reproducible performance of the clonogenic HPRT mutation assay on these cell lines.

- We have successfully performed the HPRT assay in duplicate or better on 4 BRCA1 mutant gene carrier-derived cell lines and 5 control cell lines.

- We have determined that age at draw is a significant predictor of HPRT mutation frequency in control cell lines, suggesting that the increased mutation frequency observed in vivo in aged populations is due to an intrinisic loss of function rather than simply accumulation of mutants.

- We have determined that there is a significant difference in the intrinsic viability, expressed as cloning efficiency, between the control and BRCA1 carrier-derived cell lines, with the BRCA1 carrier-derived lines showing less ability to grow in culture.

- We have determined that there is no significant difference in spontaneous mutation frequency at the HPRT locus between control cell lines and lines derived from carriers of BRCA1 mutant alleles.

- We have archived a number of HPRT-deficient mutant clones derived from cell lines from BRCA1 mutation carriers for later molecular analysis to determine whether a shift in the molecular spectrum of mutation can be detected due to their genotype at the BRCA1 locus.

Reportable Outcomes

As the work is ongoing, all reportable outcomes are still in the future. Ms. Das' Masters thesis will be completed for submission prior to the end of the spring semester, 2002. She will be encouraged to submit an abstract on this work to at least one local meeting including University of Pittsburgh Graduate School of Public Health Dean's Day and the University of Pittsburgh Cancer Institute Annual Scientific Retreat. National presentation of this work will still depend on the outcome of ongoing studies, including the results of analysis of cell lines derived from carriers of mutations in the BRCA2 gene (see **Table 1**). At least one short paper is anticipated, and perhaps as many as three, depending upon whether the age dependence observed for mutation frequency in the control lines is seen as publishable in itself, and whether the results on the BRCA2 carrier-derived lines are similar to those from the BRCA1carrier-derived lines. Several potentially fundable studies present themselves based on this work, including extension into in vivo analysis of mutation frequencies and molecular analysis of mutations in the carriers BRCA1- and 2-derived cell lines. These will be pursued as time, funding and personnel allow.

Conclusions

Our data suggest that inheritance of an inactivating mutation in the BRCA1 gene does not inherently lead to an increased frequency of spontaneous cellular mutation, and therefore a lack of DNA repair capacity is not responsible for the predisposition to breast cancer observed in carriers of BRCA1 mutations. This is in contrast to other cancer predisposing syndromes, such as AT, Fanconi anemia and Bloom syndrome, which have significantly higher frequencies of somatic mutation than normal controls (for reviews, see Grant and Bigbee, 1993; Cole and Skopek, 1992). The situation is not unlike that of HNPCC, however, in that the BRCA1 mutation is known to be genetically recessive at the cellular level, whereas it is dominant at the organismal level. The difference involves loss of heterozygosity, specifically functional loss of the remaining wild-type BRCA1 allele in the cells destined to become malignant. Thus, while our data suggest that BRCA1 heterozygous cells do not have inherently high frequencies of somatic mutation, it does not address the genomic stability phenotype of cells that have lost BRCA1 activity. We plan to complete our mutational analysis of our panel of lymphoblastoid cell lines to determine whether similar results will be found in BRCA2-derived cells. This work has been and will continue to be performed by Ms. Rubina Das, a Masters candidate in my laboratory, and will form the basis of her thesis and dissertation.

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Appendices

none